Toward a PKB Inhibitor: Modification of a Selective PKA Inhibitor by Rational Design[†]

Hadas Reuveni,[‡,§] Nurit Livnah,[*,‡,#] Tamar Geiger,[§] Shoshana Klein,[§] Osnat Ohne,[#] Ilana Cohen,[#] Moran Benhar,[§] Gary Gellerman,[#] and Alexander Levitzki[*,§]

Department of Biological Chemistry, The Silverman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel, and Peptor Ltd., Rehovot, Israel

Received March 29, 2002

ABSTRACT: Protein kinase B/Akt (PKB) is an anti-apoptotic protein kinase that has strongly elevated activity in human malignancies. We therefore initiated a program to develop PKB inhibitors, "Aktstatins". We screened about 500 compounds for PKB inhibitors, using a radioactive assay and an ELISA assay that we established for this purpose. These compounds were produced as combinatorial libraries, designed using the structure of the selective PKA inhibitor H-89 as a starting point. We have identified a successful lead compound, which inhibits PKB activity in vitro and in cells overexpressing active PKB. The new compound shows reversed selectivity to H-89: In contrast to H-89, which inhibits PKA 70 times better than PKB, the new compound, NL-71-101, inhibits PKB 2.4-fold better than PKA. The new compound, but not H-89, induces apoptosis in tumor cells in which PKB is amplified. We have identified structural features in NL-71-101 that are significant for the specificity and that can be used for future development and optimization of PKB inhibitors.

Protein kinase B/Akt (PKB)[1] is an anti-apoptotic protein kinase that has elevated activity in a number of human malignancies. PKB was originally discovered as a viral oncogene, v-Akt, in mouse T-cell lymphoma (*1*). It was later established that v-Akt is the oncogenic version of the cellular enzyme, PKBα/c-Akt1 (*2*). Sequencing of PKBα revealed homology within the kinase domains to the PKA (~68%) and PKC isozymes (~73%) (*3*), a fact that lent to its renaming as PKB. There are three cellular isoforms of PKB.

PKBα was found to be amplified or overexpressed in gastric adenocarcinomas and in a breast cancer cell line (*4, 5*). PKBβ is amplified or overexpressed in 3% of breast (*6*), 12% of pancreatic (*7*), and 15% of ovarian cancers (*6, 8*). PKBγ is overexpressed in estrogen receptor-deficient breast cancer and in androgen-independent prostate cell lines (*9*).

The mechanism of activation of PKB has been elucidated (reviewed in ref *10—12*). The second messenger PIP$_3$, which is produced by PI'-3-kinase, binds to the pleckstrin homology (PH) domain of PKB and recruits the protein to the membrane, where it is phosphorylated on residues Thr308 and Ser473 and converted to its activated form. The enzyme PDK1 phosphorylates residue Thr308 of PKB in a PIP$_3$-dependent manner (*13*). The mechanism of phosphorylation on Ser473 is controversial, and several options have been proposed. These include autophosphorylation (*14*), phosphorylation by an as yet undescribed kinase dubbed PDK2 (*15*), phosphorylation by PDK1 converted to PDK2 by binding to a specific peptide (*16*), and phosphorylation by ILK (*17*). Recently, it has been shown that phosphorylation of tyrosine residues, possibly by pp60^{c-Src}, is also required to activate PKB (*18*).

Treatment of cells with many growth factors, such as IGF-1 (*15, 19, 20*) and PDGF (*21*), leads to activation of PKB. Activation of pp60^{c-Src} (*22, 23*) also leads to the activation of PKB. Persistent activation of PKB also occurs as a result of a deletion in the gene encoding the tumor suppressor PTEN, a negative regulator of PKB (*24*). PTEN deletion is the hallmark of many advanced metastatic cancers. The overexpression of PKB in ovarian, breast, and prostate cancers is associated with a poor prognosis (*6, 9*). These findings establish the central role that PKB plays in oncogenesis and imply that inhibitors of this protein kinase could be powerful anticancer agents.

In this paper, we describe a rational approach to design novel PKB kinase inhibitors, Aktstatins, based on combinatorial and parallel chemistry methodologies. Such methodologies have been extensively used in the past few years to derive structure—activity relationships (SAR) from series and libraries of compounds, and facilitate discovery and optimization of hits and leads (25, 26). These methods allow the production of large numbers of analogues simultaneously, with a variety of modifications resulting in a diverse set of compounds based on a common structural motif. We screened a series of commercially available serine/threonine kinase inhibitors for their effects on PKB activity. Of these compounds, H-89 was chosen as a starting point for development of a PKB inhibitor, for reasons of selectivity, potency, and feasibility of chemical modification. We employed parallel synthesis to examine the effects of chemical and structural modifications to the various diversity zones of H-89, a commercially available PKA inhibitor, on the affinity and specificity of the compound analogues. To facilitate rapid screening for PKB inhibitors, we designed a nonradioactive ELISA assay of PKB. We report here on our initial success in generating a prospective lead compound for an effective PKB inhibitor. The new compound, NL-71-101, inhibited PKB activity in intact cells and led to apoptosis of an ovarian cancer cell line in which PKB is amplified.

EXPERIMENTAL PROCEDURES

General procedure for the synthesis of monosubstituted ethylenediamines on solid phase support:

One gram of 1,2-diaminoethane trityl resin (0.35 mmol/g; NOVA Biochem Ltd.) was swollen for 18 h in a reaction vessel equipped with a sintered glass bottom, and placed on a shaker.

After the sample was washed with NMP (2 times, 8 mL for 2 min), the appropriate acid (3 equiv) was preactivated in NMP (8 mL) for 3 min using PyBroP (equiv) and DIEA (6 equiv). The activated acid was transferred to a reaction vessel and shaken for 1.5 h at room temperature. Reaction completion was monitored by Kaiser test, and the solvent was removed by filtration. The resin was washed with NMP (5 times, 8 mL for 2 min) and DCM (2 times, 8 mL for 2 min), dried, and submitted to cleavage.

Piperonyl chloride was reacted directly with the resin in DCM (10 mL) in the presence of DIEA (6 equiv) for 1 h at room temperature. The workup was carried out by the same mode as for acids.

Cleavage. The dry peptidyl-resin was treated with a cold mixture of 5% TFA and 1% TIS in 10 mL of DCM. The solvent was then removed by a stream of nitrogen, and the residue was taken into diethyl ether. After centrifugation and removal of the solvent, the crude product was obtained. This was submitted to the next step without any further purification.

(i) *N*-(isoquinoline-1-carboxyamide)-ethane amine: yield (93%).

MS *m/z* (%) = 216 (M+, 100%), 1H NMR (CDCl3): δ 3.27 (t, J = 6 Hz, 2H, CH2), 3.84 (t, J = 6 Hz, 2H, CH2), 7.18 (t, J = 7 Hz, 1H), 8.13 (t, J = 7 Hz, 1H), 8.23 (d, J = 7 Hz 1H, H-5), 8.45 (m, 3H-3,4,8).

(ii) *N*-(quinoline-4-carboxyamide)-ethane amine: yield (95%).

MS *m/z* (%) = 216 (M$^+$, 100%), ^{1}H NMR (CDCl$_3$): δ 2.76 (t, J = 6 Hz, 2H, CH$_2$), 3.36 (t, J = 6 Hz, 2H, CH$_2$), 7.57 (d, J = 7 Hz, H-3), 7.68 (t, J = 7 Hz, 1H), 7.88 (t, J = 7 Hz 1H), 8.05 (d, J = 7 Hz, 1H, H-5), 8.19 (d, J = 7 Hz, 1H-8), 8.78 (bs, NH), 8.93 (s, 1 H, H-2).

(iii) *N*-(cinnoline-4-carboxyamide)-ethane amine: yield (98%).

MS *m/z* (%) = 217 (M$^+$, 100%), ^{1}H NMR (CDCl$_3$): δ 2.87 (t, J = 6 Hz, 2H, CH$_2$), 3.50 (t, J = 6 Hz, 2H, CH$_2$), 7.86 (t, J = 7 Hz, 1H), 8.07 (t, J = 7 Hz, 1H), 8.15 (d, J = 7 Hz, 1H, H-5), 8.31 (d, J = 7 Hz, 1H-8), 9.02 (bs, NH), 9.32 (s, 1 H, H-3).

(iv) *N*-(piperonylamide)-ethane amine: yield (92%).

MS *m/z* (%) = 209 (M$^+$, 100%), ^{1}H NMR (CDCl$_3$): δ 2.99 (t, J = 6 Hz, 2H, CH$_2$), 3.48 (t, J = 6 Hz, 2H, CH$_2$), 6.09 (s, 1 H, O-CH$_2$-O). 7.02 (d, J = 7 Hz, H-6), 7.40 (s, 1H-3), 7.48 (d, J = 7 Hz, 1H-5), 8.45 (bs, NH).

(v) *N*-(isoquinoline-5-carboxyamide)-ethane (*N''*-methyl)-amine: yield (89%).

MS *m/z* (%) = 280 (M$^+$, 100%), ^{1}H NMR (CDCl$_3$): δ 2.97 (m, 6H, 2CH$_3$), 3.42 (t, J = 6 Hz, 2H, CH$_2$), 3.58 (t, J = 6 Hz, 2H, CH$_2$), 7.91 (t, J = 7 Hz, H-8), 8.38 (d, J = 7 Hz, 1H, H-8), 8.47 (d, J = 7 Hz, 1H, H-6), 8.55 (d, J = 7 Hz, 1H, H-4), 8.75 (d, 1 H, H-3), 9.59 (s, 1 H, H-1).

(vi) *N*-(nicotinamide)-ethane amine: yield (91%).

MS *m/z* (%) = 166 (M$^+$, 100%), ^{1}H NMR (CDCl$_3$): δ 2.96 (t, J = 6 Hz, 2H, CH$_2$), 3.52 (t, J = 6 Hz, 2H, CH$_2$), 7.48 (t, J = 7 Hz, H-5), 8.10 (d, J = 7 Hz, 1H, H-4), 8.59, (d, J = 7 Hz, 1H, H-6), 8.77 (s, 1 H, H-1).

General Procedure for the Synthesis of Monosubstituted Ethylenediamines in Solution. Sulfonic acid was placed in a round-bottomed flask and thionyl chloride (15 equiv) was added, followed by a drop of DMF. The mixture was refluxed for 2 h and then cooled and evaporated to dryness. The powdered residue was transferred in small portions to a cooled (0°) flask containing 10 equiv of amine in methylene chloride. The mixture was stirred at room temperature for 4 h. The progress of the reaction was monitored by TLC using 25% methanol in chloroform. The mixture was evaporated and purified either by flash chromatography on silica with a gradient of 10—30% methanol in chloroform, or by MPLC using an RP-C18 column.

(i) *N*-(isoquinoline-5-sulfonamide)-ethaneamine: yield 35%.

MS *m/z* (%) = 251 (M$^+$, 100%), ^{1}H NMR (MeOD): δ 2.64 (t, J = 6 Hz, 2H, CH$_2$), 2.92 (t, J = 6 Hz, 2H, CH$_2$), 7.81 (t, J = 7 Hz, 1H, H-7), 8.36 (d, J = 7 Hz, 1H), 8.43 (d, J = 7 Hz 1H), 8.53 (d, J = 7 Hz, 1H), 8.60 (d, J = 8 Hz, 1H), 9.36 (s, 1 H, H-1).

(ii) *N*-DANSYL-ethaneamine: yield 48%

MS *m/z* (%) = 294.8 (M$^+$, 100%), ^{1}H NMR (MeOD): δ 2.53 (t, J = 6 Hz, 2H, CH$_2$), 2.85 (t, J = 6 Hz, 2H, CH$_2$), 2.85 (s, 6H, N-dimethyl) 7.24 (t, J = 8 Hz, 1H), 8.56 (m, 2H), 8.16 (d, J = 8 Hz 1H), 8.53 (d, J = 8 Hz, 1H).

(iii) *N*-(isoquinoline-8-sulfonyl)piperazine: yield 52%.

MS *m/z* (%) = 278.8 (M$^+$, 100%), ^{1}H NMR (MeOD): δ 2.81 (m, 4H,), 3.11 (m, 4H), 7.87 (t, J = 8 Hz, 1H), 8.46 (d, J = 8H 2H), 8.62 (s, 2H), 9.40 (s, 1H).

(iv) *N*-(isoquinoline-8-sulfonyl)-1,2,diaminomethylcyclohexane: yield 55%.

MS *m/z* (%) = 305.8 (M$^+$, 100%), ^{1}H NMR (MeOD): δ 1.15 (m, 4H), 1.43 (m, 4H), 2.65 (m, 1H, CH), 3.32 (m, 1H,

CH), 7.796 (t, $J = 8H$ 1H), 8.41 (d, $J = 8$ Hz 1H), 8.49 (m, 2H), 8.61 (s, 1H), 9.37 (s, 1H).

(v) *N*-(isoquinoline-8-sulfonamide)-propanamine: yield 52%.

MS *m/z* (%) = 266.8 (M$^+$, 100%), ^{1}H NMR (MeOD): δ 1.57 (dt, $J = 7$ Hz, 1H), 1.66 (dt, $J = 7$ Hz, 1H), 2.59 (t, $J = 7H$, 1H), 2.96 (m, 2H), 3.05 (t, $J = 7H$, 1H), 7.81 (t, H=8H, 1H), 7.89 (s, 1H, H-amide), 8.38 (d, $J = 8$ Hz 1H), 8.46 (d, $J = 8$ Hz, 1H) 8.56 (m, 2H), 9.36 (s, 1H).

General Procedure for Reductive Amination Using Parallel Synthesis in a 96-Well Format. Stock solutions of amines and aldehydes were prepared as 0.5 M in methanol or dimethyl sulfoxide (DMSO) depending on solubility. A total of 1.1 equiv of amine was pipetted into the wells, followed by 1 equiv of each of the various aldehydes, with accurate documentation of aldehyde ID and well position in the plate. The plate was shaken for 5 min. Then, 2 equiv of NaBH$_4$-CN were added as a freshly made 0.5 M solution in methanol, immediately followed by 0.1 equiv of acetic acid. The plate was shaken for 6 h. At the end of the reaction, the solvent was evaporated to dryness using a speedvac, and reconstituted in neat DMSO, to a final concentration of 200 mM. Identity and homogeneity of the products were evaluated by direct MS of all wells and HPLC of a representative sample of the wells.

Plasmids. (a) His$_6$-HA-Tagged PKBα. The N-terminal segment (*Eco*RI to *Not*I) of hemaglutinin (HA)-tagged PKBα, in plasmid pCMV5-HA-PKBα (*15*), was replaced with a PCR fragment encoding a His$_6$-tag before the HA tag. The 3′ primer for incorporation of the His$_6$-tag was 5′CAGAATTCGCCACCATGCATCATCATCATCATCAT-GCAGCATACCCATATGATGTGCCAG.

(b) Myristoylated/Kinase Dead PKB (KD-PKB). The C-terminal segment (*Not*I to *Bam*HI) of pCMV5-m/p-HA-PKBα (*27*) was replaced with the corresponding fragment from the kinase-dead construct, pCMV5-HA-PKBαK179A (*15*).

(c) GSK3. Plasmid pCMV5-EE-GSK3β was described by Shaw et al. (*28*).

Cell Culture and Transfection. The human embryonic kidney cell line, HEK293, the human ovarian epithelial cancer cell line, OVCAR-3 (*8*), and mouse NIH3T3 cells were cultured at 37 °C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS (Bet-Haemek, Israel). All treatments described in this report were performed in DMEM containing 10% FCS. HEK293 and NIH3T3 cells were transiently transfected using polyethyleneimine (*29*).

PKB Preparation. HEK293 cells were grown on 10-cm Petri plates and transfected with pCMV5-His$_6$-HA-PKBα. Twenty-four hours after transfection, cells were starved in serum-free medium for 20 h, stimulated with 50 ng/mL IGF-1 (Sigma) for 10 min, and lysed in 0.7 mL of cold lysis buffer (50 mM Tris-HCl pH 7.5, 1% (v/v) Triton X-100, 0.1% (v/v) 2-mercaptoethanol, 1 mM sodium orthovanadate, 20 mM sodium β-glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 μM microcystin-LR, 1 mM benzamidine, 0.2 mM AEBSF, 10 μg/mL leupeptin, 1 μg/mL aprotonin, and 10 μg/mL soybean trypsin inhibitor). The lysate was centrifuged at 4 °C for 15 min at 19000g, and the supernatant was loaded onto nickel beads (Ni-NTA agarose, Qiagen). The column was washed with buffer A (20 mM Tris-HCl, pH 7.5, 1 M KCl, 20 mM imidazole, 10% glycerol) and His-PKB was eluted with buffer B (20 mM

Tris-HCl, pH 7.5, 200 mM imidazole, 100 mM KCl, 10% glycerol). His-PKB activity was determined as described below. One unit of PKB activity is the amount of enzyme that catalyzes the phosphorylation of 1 nmol of RPRTSSF peptide in 1 min in the presence of 100 μM ATP. The specific activity of the enzyme produced was ~35 units/mg of His-PKB.

In Vitro Kinase Assays. (a) Radioactive Assay of PKB Activity. PKB activity was assayed on a 7-mer peptide RPRTSSF, which is derived from the Crosstide peptide (*30*). We verified that the peptide showed the same values of K_m and V_{max} for PKB as the parent Crosstide (*20*). In our hands, the K_m for the 7-mer or for Crosstide was 18 μM, the K_m for ATP was 40 μM, and the reaction was linear at 15 min. The reaction conditions for in vitro assay of PKB inhibition were adapted from ref *30*. The reaction mix contained 50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 1 μM PKI, 10 mM magnesium acetate, the potential inhibitory compound, 20 μM RPRTSSF peptide, 10 μM [γ^{32}P]ATP (1 μCi/assay), and 0.006 units of His-PKB. The assay was carried out in a volume of 50 μL, for 15 min at 30 °C, in 96-well plates. The reaction was terminated by addition of EGTA to a final concentration of 50 mM, and the reaction mixtures were spotted onto 6.25 cm^2 phosphocellulose strips (P81 Whatman). The strips were washed six times with 75 mM phosphoric acid (10 mL/sample) and once with acetone, air-dried, and monitored using a β-scintillation counter. The libraries of the crude inhibitory compounds were screened at a single concentration for each compound (20 μM), while purified compounds were assayed at various concentrations, and their IC$_{50}$ values were determined using the regression program.

(b) ELISA Assay of PKB Activity. We established an ELISA assay of PKB activity for high throughput purposes. 40 pmol/well of biotinylated Crosstide [K(biotin)GRPRTSS-FAEG; Sigma Israel Ltd.], dissolved in PBS, was incubated for 30 min at room temperature in streptavidin-coated 96-well plates (Steffens Biotechnische Analyzen GmbH, biotin binding capacity 18 pmol/well). Following coupling, the excess peptide was washed out. Reactions were carried out in a final volume of 50 μL/well. First, 0.006 units of His-PKB, in 50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol were placed in each well, except the blanks. Second, the inhibitory compounds were added to a final concentration of 20 μM. Finally, the reaction was started by the addition of the assay mixture (final concentrations: 50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 1 μM PKI, 10 mM magnesium acetate, and 10 μM ATP). Following agitation for 15 min at 30 °C, the reactions were stopped by addition of EDTA to a final concentration of 200 mM. Alternately, biotinylated Crosstide (1 μM) was included in the reaction mix, the reactions were performed in 96-well plates with conical wells (Sterilin), and after the reactions were terminated the contents of each well were transferred to the 96-well streptavidin plates. The plates were incubated for 30 min at room temperature, to allow coupling. The alternate protocol gave a higher signal/noise ratio. The plates were washed four times with TBST (10 mM Tris-HCl, pH 7.5, 170 mM NaCl, and 0.2% Tween-20) and blocked for 20 min at room temperature in blocking solution (1:20 dilution of low-fat milk in TBST). Phosphorylated Crosstide was detected by anti-phospho(Ser21)GSK3 (obtained from New-England BioLabs; dilution 1:1000 in

5% BSA in TBST, 45 min at RT). The wells were washed four times with TBST and incubated with peroxidase-conjugated anti-Rabbit IgG (from Jackson; dilution 1:10 000 in blocking solution) for 30 min at room temperature. Following four washes with TBST and one wash with TBS (10 mM Tris-HCl, pH 7.5, 170 mM NaCl), a color reaction was initiated by addition of a solution containing 0.5 mg/mL ABTS, 0.013% H_2O_2, 38 mM sodium hydrogen phosphate, and 31 mM citric acid. Optical densities were determined, after about 20 min, using an ELISA-Reader. Inhibitor libraries were screened either by the radioactive assay or by ELISA. H-89 was included in each assay as a positive control, at a concentration of 3 μM.

(c) Radioactive Assay of PKA Activity. PKA kinase inhibition was assayed as described by Roskoski *(31)*. Briefly, the assay contained 50 mM MOPS, pH 7.0, 10 mM magnesium acetate, 0.2 mg/mL bovine serum albumin, 10 μM [γ^{32}P]ATP (1 μCi/assay), 20 μM LRRASLG (Kemptide), and the inhibitory compound. The K_m for ATP is 25 μM. The reaction was started by the addition of 0.4 units/assay of PKA (Promega Corp.). One unit of PKA was defined by the company as the amount of enzyme required to incorporate 1 pmol of phosphate into casein in 1 min. The reaction was carried out in a volume of 50 μL/well in 96-well plates, at 30 °C for 10 min, and stopped by addition of EGTA to a final concentration of 100 mM. Twenty microliter aliquots of the assay mixture were spotted onto 2 cm^2 phosphocellulose strips. Washing and monitoring were conducted as described for PKB. The inhibitor libraries were screened in duplicate, using a single concentration of the inhibitor (1 or 5 μM, depending on the compound). Purified compounds were assayed at various concentrations, and their IC$_{50}$ values were determined using the regression program.

(d) Radioactive Assay of PKC Activity. PKC was obtained from Promega Corp. and assayed using a kit from the same manufacturer, in the presence and absence of phospholipids. The activity of PKC was determined by subtracting the activity in the absence of phospholipids from that in the presence of phospholipids. The concentration of ATP in the assay was 10 μM (K_m for ATP = 50 μM).

(e) Radioactive Assay of PKG Activity. PKG was obtained from Promega Corp., and assayed according to the manufacturer's instructions, in 40 mM Tris-HCl, pH 7.4, 20 mM MgCl$_2$, 200 μM [γ^{32}P]ATP (1 μCi/assay) using 113 μg/mL RKISASEF substrate, also from Promega Corp., in the presence of 3 μM cGMP and inhibitor. PKG was assayed at 200 μM ATP, following the manufacturer's instructions, whereas the other kinases were assayed at 10 μM ATP. Thus, the experimental IC$_{50}$ values obtained for the inhibition of PKG were corrected to values that correspond to those expected in an assay that would contain 10 μM ATP (K_m for ATP = 57 μM).

(f) Radioactive Assay of p38 Activity. HA-tagged p38 protein was immunoprecipitated, with anti-HA antibodies, from 200 μg of a lysate of UV-irradiated NIH3T3 cells that had been transiently transfected a day previously with a plasmid encoding HA-p38. The enzyme was divided into aliquots, which were incubated for 30 min at 30 °C, in the presence of 10 μM GST-ATF2 as substrate, 20 μM [γ^{32}P]ATP (5 μCi/reaction), and inhibitor. The reactions were terminated by the addition of Laemmli sample buffer (50 mM Tris-HCl, pH 6.8, 5% 2-mercaptoethanol, 3% sodium dodecyl sulfate, and 0.5 mg/mL bromophenol-blue), and the
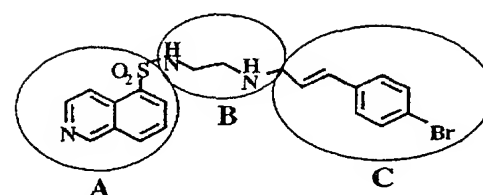

FIGURE 1: Structure of H-89, showing the division into diversity domains. Libraries were designed to explore the contribution of each domain to potency and specificity.

reaction mixtures were separated by SDS−PAGE. The amount of phosphorylated substrate was quantified using a phosphor-imager (Fujifilm, FLA 300).

Immunoblotting. HEK293 cells were treated with inhibitors 48 h following transfection with pCMV5-EE-GSK3. After 4 h incubation with the inhibitor, cells were lysed in boiling Laemmli sample buffer. Aliquots of cell extracts containing equal amounts of protein were resolved by 10% SDS−PAGE and electroblotted onto nitrocellulose filters. The membranes were blocked with blocking solution, incubated with primary antibodies diluted in 5% BSA in TBST overnight at 4 °C, and then with horseradish peroxidase-conjugated secondary antibodies diluted in blocking buffer for 75 min at room temperature. Immunoreactive bands were visualized using ECL, and quantitated using the NIH-Image program.

Antibodies against phospho-Akt (Thr308), phospho-Akt (Ser473), and phospho-GSK3α (Ser21) were obtained from New England BioLabs. Antibodies against GSK3β were from Transduction Laboratories. Anti-Akt1/2 (H-136) was from Santa Cruz Biotechnology.
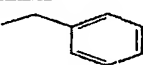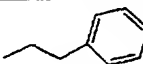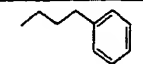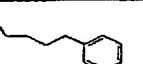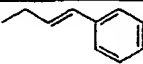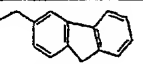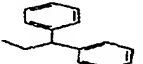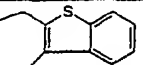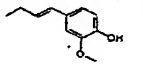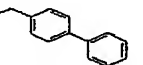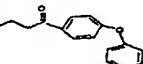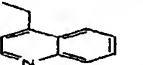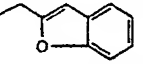
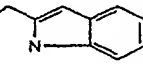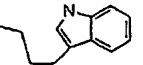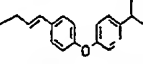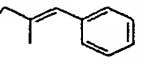*Apoptosis Assays.* OVCAR-3 cells were seeded in 10-cm Petri plates (2 × 10^6 cells/plate) in DMEM containing 10% FCS. Twenty-four hours later, NL-71-101, NL-71-101B, or H-89 were added to various concentrations. Following 40 h of treatment, cells were harvested, washed twice with PBS, and stained with Annexin-V-FLOUS + PI according to the manufacturer's instructions (Boehringer Mannheim). Cells were counted with a FACS.
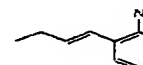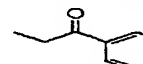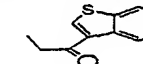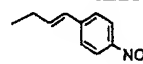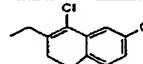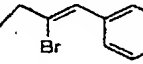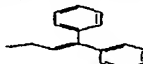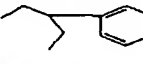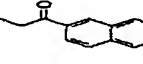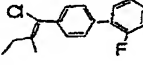
For DNA fragmentation analysis, cells were incubated with PI solution (50 μg/mL PI, 0.1% sodium citrate, 0.1% Triton X-100, 100 μg/mL RNAse, in PBS), and the proportion of the population in the sub-G1 phase was quantified by FACS analysis.

RESULTS

H-89 as an Initial Lead Compound. Since there are no known inhibitors of PKB, we screened a variety of commercial Ser/Thr protein kinase inhibitors, in an attempt to identify structural motifs that would assist in library design (data not shown). Several molecules inhibited PKB in the low micromolar range. A combination of affinity and specificity considerations, as well as analysis of the structural properties of these compounds, led us to select H-89 as the basis for library design. The structure of H-89 makes it a suitable candidate for SAR study using combinatorial chemistry, since it allows diversity in many regions of the molecule (Figure 1). In the core (A, in Figure 1), the 5-isoquinoline moiety can be replaced with various bicyclic and aromatic residues, and the sulfonamide group can be replaced with carbonyl and other similar moieties. The ethylenediamine bridge (B, in Figure 1) can vary in length, hydrogen bonding properties, and substitution. Finally, the

Table 1· SAR from Screening Various C Moieties with 5-Isoquinoline-8-sulfonamide ethylenediamine

Structure	% inhibition PKB at 20μM	% inhibition PKA at 5 μM	Structure	% inhibition PKB at 20μM	% inhibition PKA at 5 μM
	68	51		85	57
	86	49		75	54
	61	91		50	48
	88			94	
	88			53	39
	92			78	10
	76	44		88	
	67	55		81	65
	87			83	44
	50			92	59
	55	56		22	_13
	12			33	17
	54	53			
	66	80			
	84				
	76	61			
	82	38			
	89				

cinnamoyl moiety (C, in Figure 1) can be modified to a large variety of structures for the evaluation of the optimal properties of this region.

Structure—Activity Study. We examined the effects of structural and chemical modifications in the diversity regions of H-89 defined above. In most libraries, starting materials

FIGURE 2: A, B, and C moieties used in combinatorial arrays for the SAR studies of H-89 analogues.
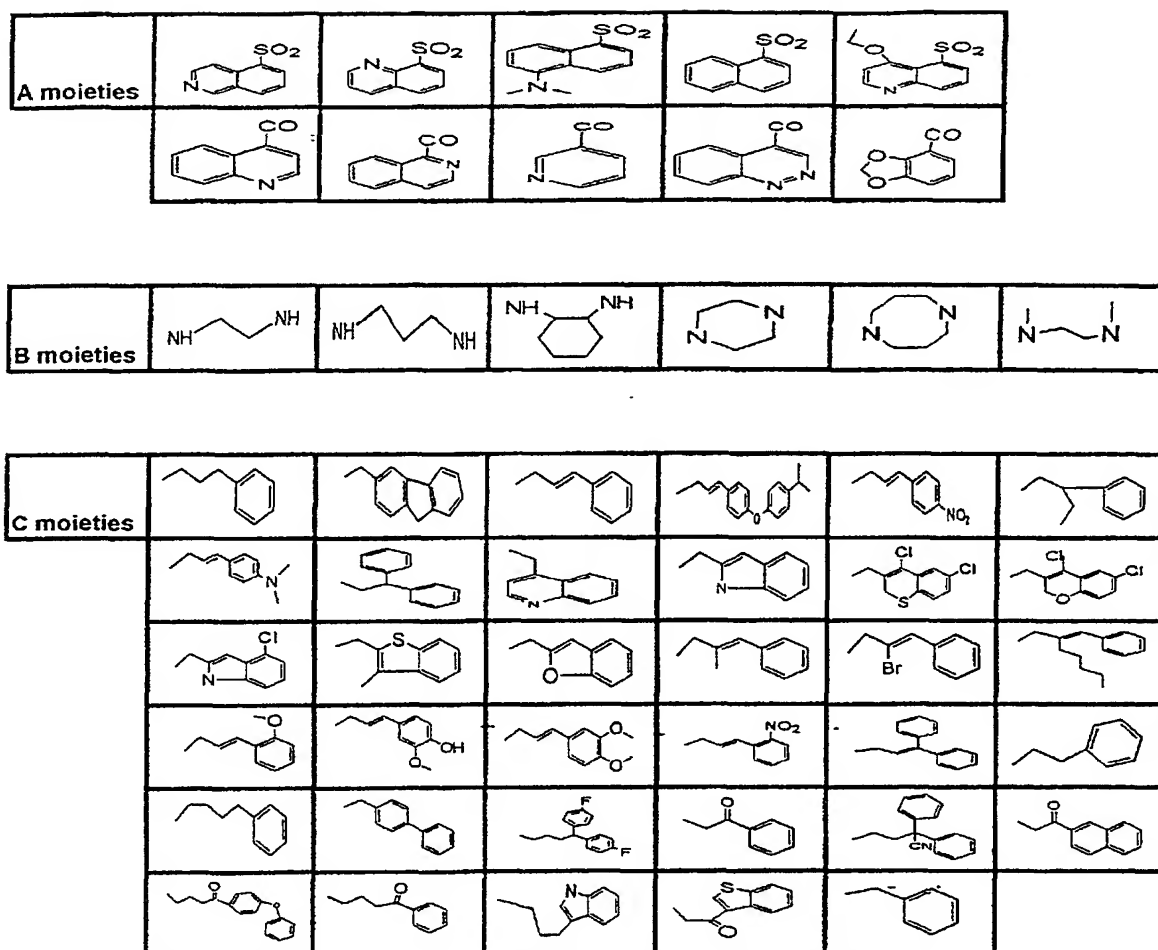
were prepared from sulfonic or carboxylic acids reacted with diamines to produce sulfonamides or amides, respectively. These starting materials were subjected to parallel synthesis using alkylation or reductive amination, with the appropriate halides or aldehydes, respectively. The libraries were screened for both PKB and PKA inhibition, to assess the contribution of each structural modification to the inhibition of each enzyme. A simultaneous SAR study for both PKA and PKB was necessary, since the initial lead compound, H-89, is a PKA antagonist, and the SAR study was directed mainly at identifying the specific enzyme—ligand interactions that are not common to both PKA and PKB. Such interactions could be modulated by structural features that either adversely affect the interaction with PKA ("irritants") without significantly affecting the interaction with PKB, or selectively improve binding to PKB.

H-89 analogues for SAR study were synthesized in parallel in a 96-well format, using solution chemistry. In the initial tests, the crude compounds (~80−85% pure) were tested at 20 μM in the assays of PKB activity, and at 1 or 5 μM (depending on the compound; see Table 1) in the PKA assays. In every assay, H-89 itself was included as a reference for comparison.
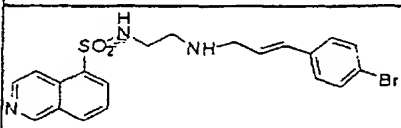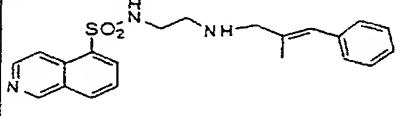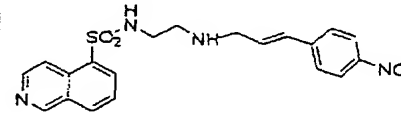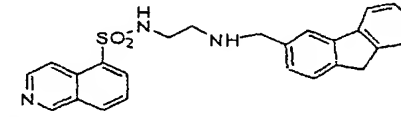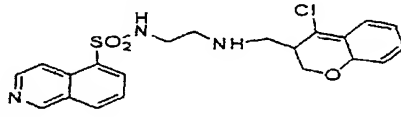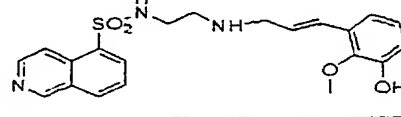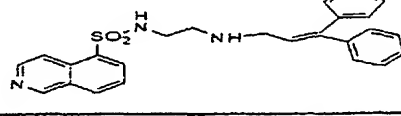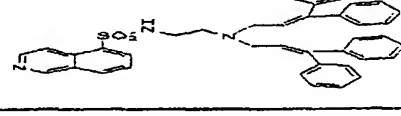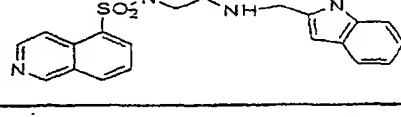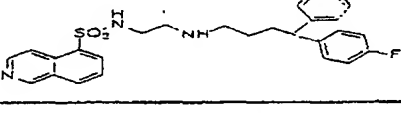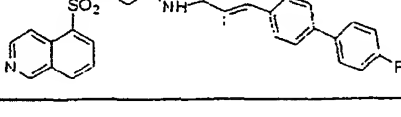
In preliminary studies, we built a small library based on aliphatic residues in C to examine the necessity for the aromatic moiety that H-89 bears in this region. Very low inhibition was observed by this library. Several single compounds with similar structures were synthesized later to confirm these observations, and these also proved to be very poor inhibitors (data not shown). At this stage, we concluded that interaction with a highly hydrophobic feature in region C is very important.

Next, we synthesized a small library to evaluate the chemistry of the bond connecting regions C and B. An amine bond (as in H-89) and an amide bond were synthesized and compared, for three different bicyclic A cores and three different C moieties (data not shown). This series clearly showed that an amide bond is less favorable for the inhibition of PKB than an amine. We also synthesized a small library with a urea bond connecting these two regions. Again, significantly lower inhibition was observed by these compounds than by their amine analogues.

On the basis of these results, we designed several libraries with an amine connecting part C and B, and a bulky hydrophobic residue in part C. The libraries were composed of combinatorial arrays of the A, B, and C moieties depicted in Figure 2. SAR analysis of these libraries showed that changes in the structures of A or B adversely affected the inhibition of PKB (data not shown). On domain A, any replacement of the 5-isoquinoline-sulfonamide moiety, including various other quinolines, abolished the inhibition. These results imply that the nitrogen at position 5 is significant for the inhibition. Substitutions on the A core also diminished the inhibition, possibly implying that the binding

Table 2: IC$_{50}$ Values of Purified H-89 Analogues

Structure	Inhibitor	IC$_{50}$(PKB), μM	IC$_{50}$(PKA), μM
	H-89	2.5	0.035
	NL-71-101	2	0.12
	NL-71-99a	2.6	0.14
	NL-71-99b	6.5	0.16
	NL-71-99d	28	0.52
	NL-71-101c	9	0.4
	NL-71-101	3.7	9
	NL-71-101b	>100	13
	NL-71-99c	>100	12
	NL-71-133a	5.6	8.5
	NL-71-135a	13	2.8

pocket is very tight at this region. On the bridge B, elongation from two to three carbons diminished activity, as did substitution on the chain. Placement of secondary amine derivatives in region B adversely affected the inhibition:

Piperazine or *N,N*-dimethylethylamine led to significantly reduced inhibition, whereas homopiperazine eliminated the inhibitory effect. These results imply that the amino protons are important for the interaction, possibly through hydrogen bonding, and that the distance and relative positions of these two basic centers are crucial. The possibility that only one of the hydrogen bonds actively participates in the interaction needs to be further elucidated, by eliminating only one of the basic centers.

The most significant SAR was observed in the moieties tested for part C, where for the same A and B, we observed a significant effect of the nature of C on potency and specificity (Table 1). The performance of compounds derived from 5-isoquinoline-sulfonamide-ethylenediamine, in which various moieties were placed in region C, ranged from almost no PKB inhibition to inhibition of over 90% at 20 μM. Significant variation was observed in the effects on PKA as well. In several wells, the inhibition of PKA was significantly decreased. Overall, these results indicated that the interaction with region C is important for both enzymes, and furthermore, that an "irritant" interaction with PKA might exist in region C, which does not significantly affect the interaction with PKB. Specifically, the initial results suggested that an additional substitution at the carbon bearing the aromatic residue in region C is unfavorable for PKA inhibition, while its effect on PKB inhibition is negligible.

Several promising compounds were selected for resynthesis, purification, and further characterization, and accurate IC$_{50}$ values were evaluated for them. In addition, we synthesized, purified, and characterized several other compounds with an additional hydrophobic interaction in region C, to confirm that this position distinguishes the interaction of the compounds between PKB and PKA. The IC$_{50}$ values obtained in radioactive assays using the purified compounds are depicted in Table 2. These results confirm that this position is indeed a PKA "irritant", shifting its IC$_{50}$ value from less than 50 nM to several micromolar, while its effect on PKB is minor.

Of all the compounds examined, NL-71-101 showed the best combination of selectivity and activity against PKB. As shown in Figure 3, NL-71-101 was almost as potent as H-89 against PKB (IC$_{50}$ of NL-71-101 = 4 μM, of H-89 = 2.5 μM), but had lost potency against PKA (IC$_{50}$ of NL-71-101 = 9 μM, of H-89 = 0.035 μM). Furthermore, NL-71-101 had also lost potency against two other Ser/Thr kinases, PKC and PKG as compared to H89 and was inactive toward p38^{MAPK}, even at concentrations of 100 μM (Table 3). Thus, using SAR studies, we have converted H-89, which is a 70-fold more potent inhibitor of PKA than of PKB, into a compound that is dramatically reduced in its potency against PKA, without affecting the degree of PKB inhibition.

NL-71-101 is an ATP-competitive inhibitor. The kinetics of inhibition of PKB and PKA by NL-71-101 were examined using radioactive kinase assays. Enzyme activities were assayed at several concentrations of NL-71-101, over a range of ATP concentrations (5–50 μM). Reciprocal plots of reaction velocity versus ATP concentration intersected on the *Y*-axis (Figure 4), showing that NL-71-101 is competitive with ATP.

NL-71-101 Inhibits Phosphorylation of the PKB Substrate, GSK3, in Intact Cells. To examine the effect of NL-71-101 on the activity of PKB in intact cells, HEK293 cells were
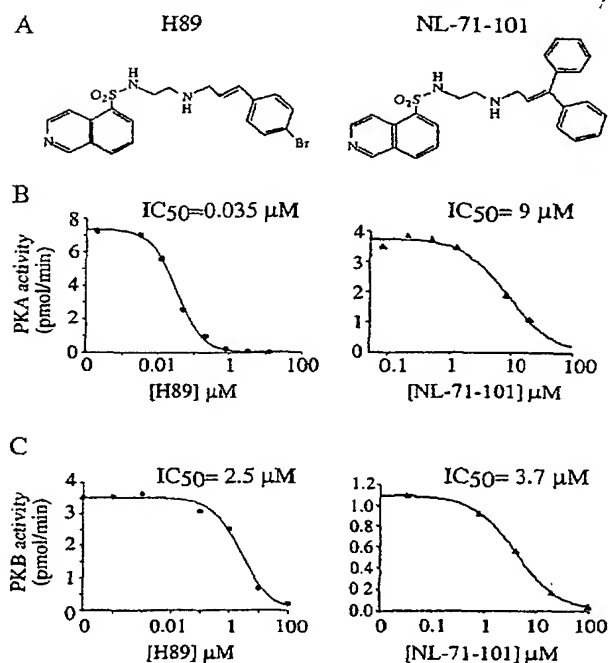
FIGURE 3: Structures and inhibition potency of H-89 and NL-71-101 in cell free assays. (A) Structures of H-89 and NL-71-101. (B) In vitro inhibition of PKA activity by the inhibitors. (C) In vitro inhibition of PKB activity by the inhibitors.

Table 3: IC$_{50}$ Values of NL-71-101 and H-89 for Several Related Kinases[a]

	IC$_{50}$ values (μM)	
enzyme	NL-71–101	H-89
PKB	3.7	2.5
PKA	9	0.035
PKG	36 (10.8)	0.88 (0.29)
PKC	104	15
p38	$\gg$100	not determined

[a] Values in parentheses represent calculated IC$_{50}$ values corrected to 10 μM ATP.

transiently transfected with a plasmid encoding the PKB substrate, GSK3. Forty-eight hours later, the transfected cells were treated with NL-71-101 at various concentrations, for 4 h. IGF-1 (10 ng/mL) was added and 10 min later lysates were prepared, separated by SDS–PAGE, and subjected to Western analysis. Phosphorylation of GSK3 was detected using a specific antibody to GSK3 phosphorylated at residue Ser21, and the intensities of the bands corresponding to phosphorylated GSK3β were normalized to the intensities of the total (phosphorylated plus unphosphorylated) GSK3β bands, detected using anti-GSK3β antibody (Figure 5). NL-71-101 inhibited GSK3 phosphorylation in a dose-dependent manner with an IC$_{50}$ ∼ 25 μM (Figure 5). The effect of NL-71-101 treatment of HEK293 cells on GSK3 phosphorylation levels was comparable to the specific inhibition of PKB activity by a dominant negative, myristoylated kinase dead PKB mutant (KD-PKB; Figure 5). Note that the amount of GSK3 was low in the cells cotransfected with both GSK3 and KD-PKB plasmids; this may be due to competition for transcription/translation factors when the two genes were overexpressed simultaneously. NL-71-101 also inhibited the phosphorylation of residue Ser 473 of PKB, a site that is

FIGURE 4: Kinetics of PKB and PKA kinase inhibition by NL-71-101. The activities of PKB and PKA were determined using the radioactive assays, as descr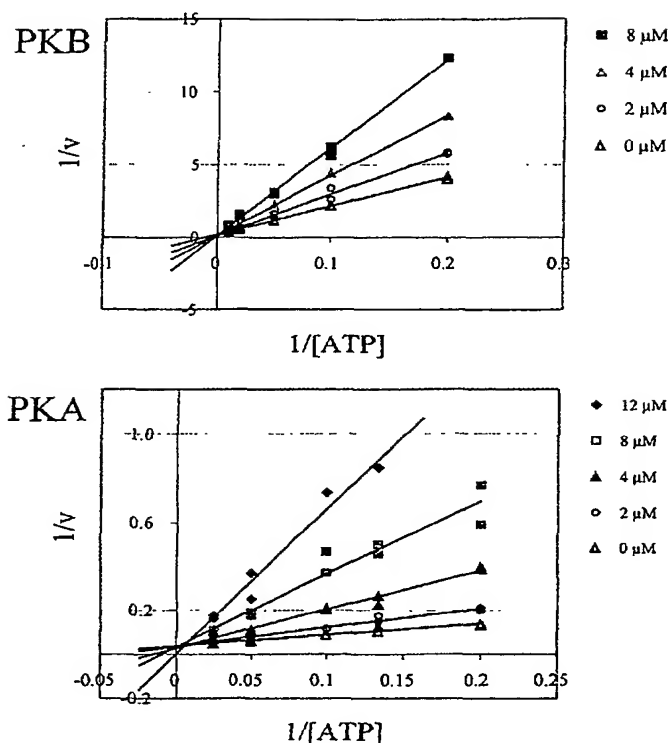ibed in Materials and Methods. The reactions were performed in the presence of a fixed concentration of the 7-mer peptide substrate (20 μM), varying concentrations of ATP (5, 7.5, 10, 20, 50 μM), and varying concentrations of NL-71-101 (0, 2, 4, 8, 12 μM).
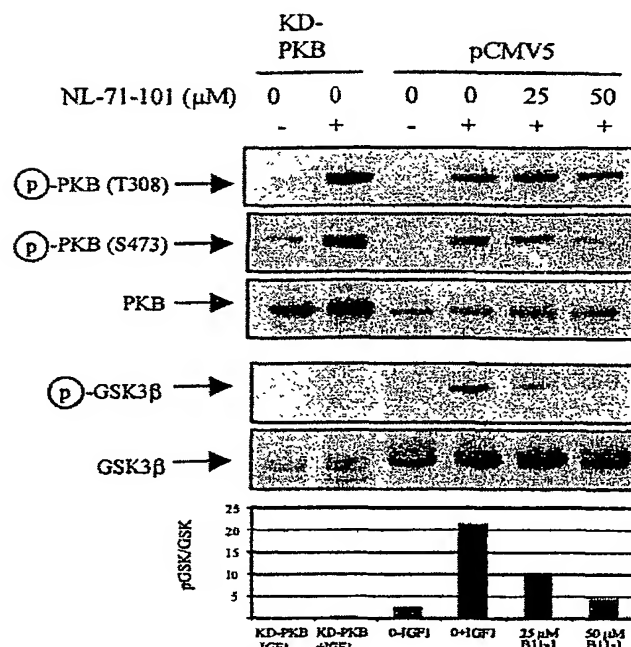

FIGURE 5: Treatment with NL-71-101 inhibits GSK3 phosphorylation in HEK293 cells. HEK293 cells were transiently transfected with a GSK3 encoding plasmid and treated with NL-71-101 at the indicated concentrations for 4 h, or transiently cotransfected with both the GSK3 plasmid and another plasmid encoding the dominant negative PKB mutant, myr-KD-PKB. Cells were then exposed to IGF-1 for 10 minutes, to activate PKB. Lysates were prepared and subjected to Western analysis, using anti-phospho(Thr308)Akt1-(PKBα), anti-phospho(Ser473)Akt(PKB), or anti-phospho(Ser21)-GSK3α antibodies. The latter antibody recognized both α and β isoforms of GSK3, when phosphorylated. Following stripping, the blots were treated with anti-Akt1/2(PKBα/β) or anti-GSK3β antibodies. The lower panel shows the quantitation of phosphorylated GSK3β, normalized to total GSK3β levels.

believed to be subject to autophosphorylation (*14*). There was a slight effect on phosphorylation of residue Thr308. This might be due to preclusion of enzyme activation due to binding of the inhibitor, or to nonspecific effects of the inhibitor. In summary, NL-71-101 affects the ability of endogenous PKB to phosphorylate its substrate, GSK3, in intact cells.

NL-71—101 Induces Apoptosis. Since PKB is an anti-apoptotic kinase, we investigated the effects of NL-71-101 on apoptosis. We treated OVCAR-3 ovarian carcinoma cells, in which PKB is strongly amplified (*8*), with NL-71-101 at various concentrations. Apoptosis was assessed using both annexin-V staining of phosphatidyl-serine residues on the cell surface (an indicator of early apoptosis; Figure 6A) and propidium iodide staining of the sub-G1 cell population (which gains prominence later in apoptosis; Figure 6B). Stained cells were counted by FACS. Treatment of OVCAR-3 cells with NL-71-101 led to a dose-dependent increase in apoptosis (Figure 6C). A significant increase in the proportion of apoptotic cells was evident after treatment with 50 μM NL-71-101, and more than half of the cells were apoptotic after treatment with 100 μM NL-71-101 (Figure 6). Thus, NL-71-101 caused intact OVCAR-3 cells to undergo apoptosis.

To assess whether the apoptosis of OVCAR-3 cells in response to NL-71-101 was due to a possible general toxic effect of the compound rather than due to inhibition of PKB, we compared NL-71-101 treatment with that of a related

compound, NL-71-101B, which might have been expected to have comparable overall toxic properties. NL-71-101B, although similar to NL-71-101 in structure, did not inhibit PKB in vitro, up to a concentration of 100 μM (Table 1). As can be seen in Figure 6B, neither did NL-71-101b lead to apoptosis of OVCAR-3 cells. Thus, there is a correlation between the ability of a compound to inhibit PKB in vitro, and its ability to induce apoptosis upon treatment of intact cells.

Since NL-71-101 inhibits both PKA and PKB activities (Figure 3), we asked whether the induction of apoptosis in OVCAR-3 cells was a consequence of PKA or PKB inhibition. To this end, we compared the effects of NL-71-101 with those of its parent, the much more selective PKA inhibitor, H-89. We argued as follows: If inhibition of PKA activity causes apoptosis of OVCAR-3 cells, H-89 should lead to apoptosis at concentrations at which it inhibits PKA. If, on the other hand, inhibition of PKB activity causes apoptosis, H-89 should only lead to apoptosis at the much higher concentrations at which it also inhibits PKB. NL-71-101 inhibits PKA in vitro with an IC$_{50}$ of 9 μM (Figure 3), and leads to significant apoptosis of cells at 50 μM (Figure 6). Since H-89 inhibits PKA in vitro with an IC$_{50}$ of 0.035 μM, we might expect apoptosis to occur at concentrations of 0.2 μM (i.e., [50 × 0.035/9] μM), had PKA been involved. In fact, up to concentrations of 20 μM, H-89 had no effect on apoptosis of OVCAR-3 cells. Apoptosis was induced at

A

B

C

μM inhibitor
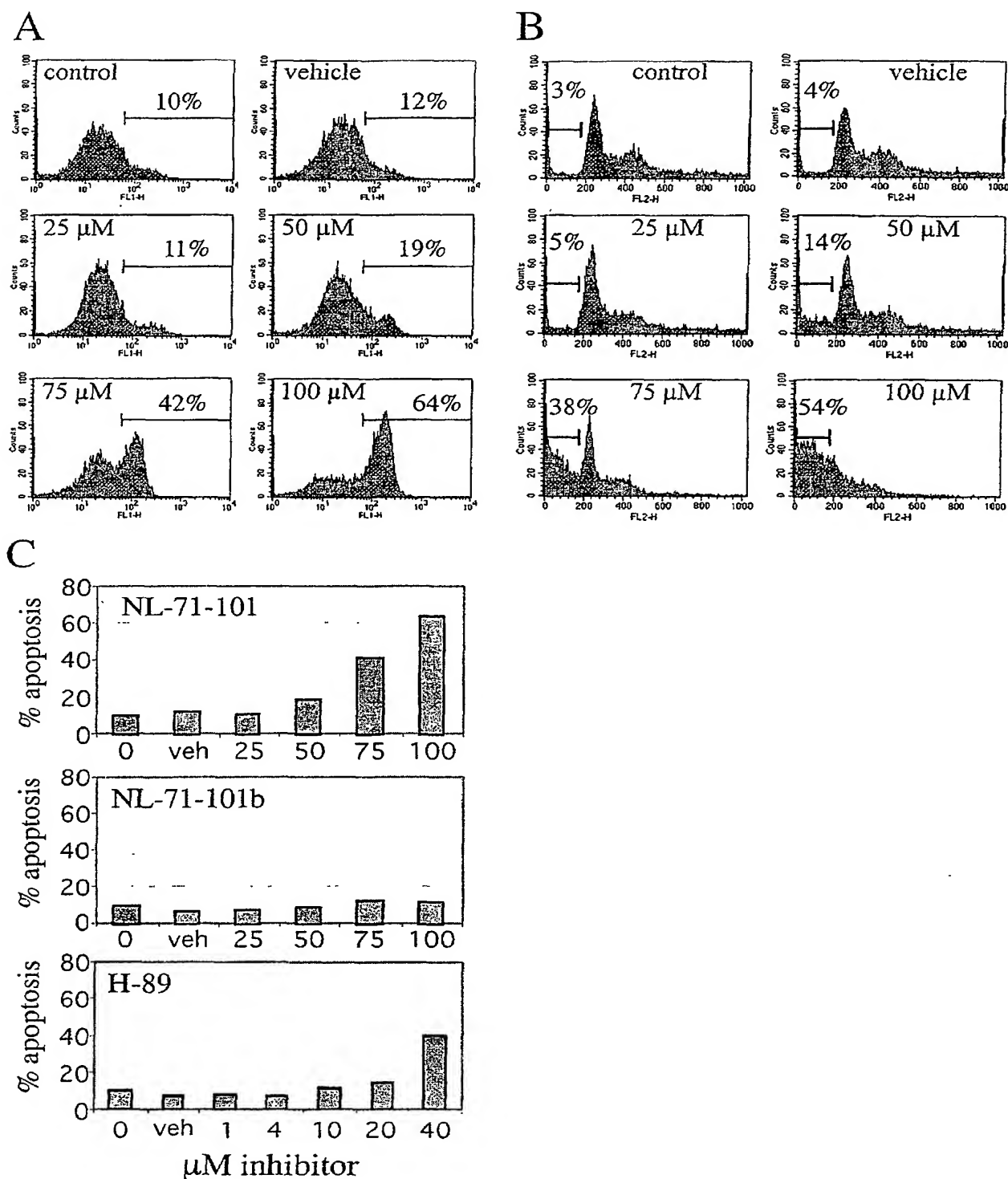
FIGURE 6: Treatment with NL-71-101 induces apoptosis of OVCAR-3 cells. OVCAR-3 cells were exposed to NL-71-101 at the indicated concentrations for 40 h in medium containing 10% FCS. (A) Cells were stained with annexin-V, and the proportion of the apoptotic population was quantified by FACS analysis. (B) Cells were stained with propidium iodide and the proportion of the sub-G1 population was determined by FACS analysis. (C) Graphic presentation of dose-dependent apoptosis of OVCAR-3 cells following 40 h of treatment with NL-71-101 (summary of section A), NL-71-101B and H-89, using annexin-V staining.

40 μM H-89, at which concentrations H-89 would be expected to inhibit PKB in intact cells, by a similar argument. It thus appears that induction of apoptosis by NL-71-101 correlates with inhibition of PKB, rather than with inhibition of PKA.

DISCUSSION

Recognizing the pivotal role PKB plays in oncogenesis, we have embarked on an attempt to generate PKB inhibitors as prospective antitumor agents. The strategy adopted in the

present study was based on the assumption that the high degree of homology between PKB and PKA should enable one to develop PKB inhibitors from selective PKA inhibitors. Our initial screen of known Ser/Thr kinase inhibitors indeed showed that H-89 is a relatively potent inhibitor of PKB, albeit much more potent (70-fold) against PKA. H-89 is an attractive starting point for drug development: The modular structure of H-89 allowed us to employ parallel chemistry and SAR to identify a moiety, the cinnamoyl tail, which could be modified to change the selectivity of the compound while retaining its affinity for PKB. Furthermore, we developed an ELISA assay to allow high throughput testing of potential inhibitors. We have succeeded in reversing the selectivity of H-89 toward PKA/PKB, arriving at a compound, NL-71-101, which is slightly more potent against PKB than PKA. Interestingly, NL71-101 inhibits PKG with similar potency to its inhibition of PKA but is a poor inhibitor of PKC (Table 3), in contrast to H89 which inhibits PKG 10-fold less well than PKA (Table 3). Also, NL 71-101 inhibits PKB 26-fold better than its inhibitory effect on PKC, whereas H89 inhibits PKB only 6-fold better than its inhibition of PKC (Table 3).

The effects of NL-71-101 on the viability of intact cells are consistent with its function as a PKB inhibitor. We have shown, using the easily transfectable HEK293 cell line, that NL-71-101 inhibits endogenous PKB, leading to a reduction in phosphorylation of exogenous GSK3 (Figure 5). NL-71-101 induced apoptosis in two carcinoma cell lines in which PKB is amplified (7), namely, the ovarian carcinoma line OVCAR-3 (Figure 6) and the pancreatic carcinoma line PANC-1 (data not shown). These data validate our postulation that a suitable PKB inhibitor should be effective in treatment of cancers in which the activity of PKB is enhanced.

PKB represses a number of apoptotic pathways (*10−12*). A recent report has shown that PKB phosphorylates and inactivates apoptosis signal-regulating kinase 1 (ASK1), an upstream activator of the stress-activated MAP kinase, p38 (*32*). We have preliminary evidence that this may be one pathway whereby NL-71-101 induces apoptosis, as we saw dose-dependent phosphorylation of p38 following treatment of OVCAR-3 cells with NL-71-101 (data not shown). We cannot, however, rule out the possibility that NL-71-101 led to general toxic effects and hence to stress pathway activation, particularly in light of the high concentrations of the compound to which the cells were exposed. NL-71-101 acts at high concentrations, but it is a promising lead compound for further development of high affinity, selective PKB inhibitors.

The crystallographic structure of PKA with H89 was reported (*33*), showing that H89 occupies the ATP binding site of the kinase. Based on this data and on our kinetic studies, showing that NL-71-101 functions as an ATP competitive inhibitor (Figure 4), further studies are currently being performed with the aim to improve the affinities of NL-71-101 analogues towards the PKB kinase domain.

REFERENCES

1. Staal, S. P., and Hartley, J. W. (1988) *J. Exp. Med. 167*, 1259−64.
2. Bellacosa, A., Testa, J. R., Staal, S. P., and Tsichlis, P. N. (1991) *Science 254*, 274−7.
3. Jones, P. F., Jakubowicz, T., Pitossi, F. J., Maurer, F., and Hemmings. B. A. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4171−5.
4. Staal, S. P. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5034−7.
5. Jones, P. F., Jakubowicz, T., and Hemmings, B. A. (1991) *Cell Regul. 2*, 1001−9.
6. Bellacosa, A., de Feo, D., Godwin, A. K., Bell, D. W., Cheng, J. Q., Altomare, D. A., Wan, M., Dubeau, L., Scambia, G., Masciullo, V., et al. (1995) *Int. J. Cancer 64*, 280−5.
7. Cheng, J. Q., Ruggeri, B., Klein, W. M., Sonoda, G., Altomare, D. A., Watson, D. K., and Testa, J. R. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 3636−41.
8. Cheng, J. Q., Godwin, A. K., Bellacosa, A., Taguchi, T., Franke, T. F., Hamilton, T. C., Tsichlis, P. N., and Testa, J. R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 9267−71.
9. Nakatani, K., Thompson, D. A., Barthel, A., Sakaue, H., Liu, W., Weigel, R. J., and Roth, R. A. (1999) *J. Biol. Chem. 274*, 21528−32.
10. Downward, J. (1998) *Curr. Opin. Cell. Biol. 10*, 262−7.
11. Chan, T. O., Rittenhouse, S. E., and Tsichlis, P. N. (1999) *Annu. Rev. Biochem. 68*, 965−1014.
12. Kandel, E. S., and Hay, N. (1999) *Exp. Cell Res. 253*, 210−29.
13. Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B., and Cohen, P. (1997) *Curr. Biol. 7*, 261−9.
14. Toker, A., and Newton, A. C. (2000) *J. Biol. Chem. 275*, 8271−4.
15. Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B. A. (1996) *EMBO J. 15*, 6541−51.
16. Balendran, A., Casamayor, A., Deak, M., Paterson, A., Gaffney, P., Currie, R., Downes, C. P., and Alessi, D. R. (1999) *Curr. Biol. 9*, 393−404.
17. Delcommenne, M., Tan, C., Gray, V., Rue, L., Woodgett, J., and Dedhar, S. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 11211−6.
18. Chen, R., Kim, O., Yang, J., Sato, K., Eisenmann, K. M., McCarthy, J. B., Chen, H., and Qiu, Y. (2001) *J. Biol. Chem. 276*, 31858−62.
19. Dudek, H., Datta, S. R., Franke, T. F., Birnbaum, M. J., Yao, R., Cooper, G. M., Segal, R. A., Kaplan, D. R., and Greenberg, M. E. (1997) *Science 275*, 661−5.
20. Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A. (1995) *Nature 378*, 785−9.
21. Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Tsichlis, P. N. (1995) *Cell 81*, 727−36.
22. Datta, K., Bellacosa, A., Chan, T. O., and Tsichlis, P. N. (1996) *J. Biol. Chem. 271*, 30835−9.
23. Liu, A. X., Testa, J. R., Hamilton, T. C., Jove, R., Nicosia, S. V., and Cheng, J. Q. (1998) *Cancer Res. 58*, 2973−7.
24. Wu, X., Senechal, K., Neshat, M. S., Whang, Y. E., and Sawyers, C. L. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 15587−91.
25. Thompson. L. A., and Ellman, J. A. (1996) *Chem Rev. 96*, 555−600.
26. Dole, R. E., and Nelson, K. H. (1999) *J. Comb. Chem. 1*.
27. Andjelkovic, M., Alessi, D. R., Meier, R., Fernandez, A., Lamb, N. J., Frech, M., Cron, P., Cohen, P., Lucocq, J. M., and Hemmings, B. A. (1997) *J. Biol. Chem. 272*, 31515−24.
28. Shaw, M., Cohen, P., and Alessi, D. R. (1997) *FEBS Lett. 416*, 307−11.
29. Boussif, O., Lezoualc'h, F., Zanta, M. A., Mergny, M. D., Scherman, D., Demeneix, B., and Behr, J. P. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 7297−301.
30. Alessi, D. R., Caudwell, F. B., Andjelkovic, M., Hemmings, B. A., and Cohen, P. (1996) *FEBS Lett. 399*, 333−8.
31. Roskoski, R., Jr. (1983) *Methods Enzymol. 99*, 3−6.
32. Kim, A. H., Khursigara, G., Sun, X., Franke, T. F., and Chao, M. V. (2001) *Mol. Cell Biol. 21*, 893−901.
33. Engh, R. A., Girod, A., Kinzel, V., Huber, R., and Bossemeyer, D. (1996) *J. Biol. Chem. 271*, 26157−64.